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Identification of HNP3 as a tumour marker in CD4+ and CD4– lymphocytes of patients with cutaneous T-cell lymphoma

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ABSTRACT

Cutaneous T-cell lymphomas (CTCL) are characterized by malignant proliferation of skin homing T-cells. Although prognosis is generally good, reliable markers are needed to identify patients at risk for a more aggressive course. ProteinChip (SELDI) technology was used as a tool for the discovery of protein patterns in lymphocytes from patients with CTCL ($n = 25$) and unaffected controls ($n = 25$). Lymphocytes were separated in CD4+ and CD4– fractions by magnetic cell sorting (MACS). Each whole protein extract was analysed by ProteinChip technology. The resulting protein profiles were submitted for bioinformatic analysis including a clustering algorithm, a rule extraction, a rating and a rule-base construction step. For the generated combined rule base for the CD4– cell fraction, both the sensitivity and specificity for the prediction of CTCL reached 96%, while for the CD4+ fraction they were 92% and 84%, respectively, for sensitivity and specificity. The most significant peak at 3489 Da could be identified as HNP3, an α-defensin, by immunocapturing. These results open up both the possibility for the use of this protein signature, especially HNP3, to more effectively monitor and screen CTCL, and the avenue to identify the other relevant peaks for a better understanding of the development of this tumour.

1. Introduction

Malignant lymphomas can affect the integument primarily and secondarily. If no extracutaneous manifestations can be detected with routine staging methods, a primary cutaneous lymphoma can be assumed. The special status of cutaneous lymphomas results from the skin associated control circuit of lymphocytes that recycle between skin and lymph node. The majority of cutaneous lymphomas are classified as T-cell lymphomas. More than 90% of the T-cell lymphomas are represented by mycosis fungoides (MF) and the leukaemic variant known as Sézary syndrome (SS). Cutaneous T-cell lymphomas (CTCL) of MF type start mostly in middle adulthood and have an incidence of 0.4/100,000 individuals/year in the US [1] but this is on the increase. CTCL is classified as a peripheral T-cell lymphoma of low malignancy with a

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prolonged indolent course. Prognosis depends mainly on the stage and severity of skin involvement and lymph node status. At the beginning of the disease histological diagnosis can be especially difficult. While life expectancy in patients with skin involvement T1 is not reduced at all, the 10-year survival rate in patients with T3 and T4 is as low as 40%. At this time, clear cut prognosis for an individual case is not possible [2].

Despite enormous efforts only a few tumour disease relevant markers have been established that can be used for early diagnosis or for a better therapy in malignancies [3]. This is despite the fact that new high parallel genomic and proteomic techniques have been established in the last few years. Up to now, there is also no highly specific tumour marker for CTCL although an increase of lactate dehydrogenase (LDH) is associated with a worse prognosis for the patient in tumour stage or erythrodermia [4]. Although numerous potential candidates have been investigated, none are yet used routinely in clinical settings. One candidate marker is neopterin, which are pyrazoino-pyrimidine molecules that are produced after IFN-γ stimulation and indicate activation of the cellular immune response [5]. Neopterins were elevated in patients with higher stages of CTCL's compared to patients with psoriasis and atopic eczema, but not in patients with SS where they were only slightly elevated. Increased neopterin levels are seen in many other malignant diseases and are not specific to CTCL. Another candidate is the α chain of soluble IL-2R (sIL-2R). Its concentration was found to be increased in advanced tumour stages of CTCL. The correlation between sIL-2R and the severity of the skin and lymph node status was better for sIL-2R than for LDH or the β2-microglobulin [6].

For the discovery of new biomarkers at the proteomic level, surface enhanced laser desorption/ionization-mass spectrometry (SELDI-MS)-based ProteinChip technology is one of the most promising techniques [7]. This technology makes use of affinity surfaces to retain proteins based on their physico-chemical characteristics, followed by direct analysis by time of flight mass spectrometry (TOF-MS) [8]. Thus, proteins being retained on chromatographic surfaces can be easily purified from contaminants such as buffer salts or detergents, thus eliminating the need for pre-separation techniques, as required with other MS techniques. Furthermore, the low sample requirements of this technique are ideal for small biopsies, microdissected tissue or cell subfractions of blood [9].

Until now, biomarker discovery with the ProteinChip technology was mostly done by analyzing body fluids like serum or urine, as body fluid analyses are fast and easy to perform by direct application on the arrays. Nevertheless, it is known that intra-individual changes in serum are high. Hence, biomarkers responsible for the genesis and progression of cancer must be present at a high level to be observed above normal changes [10]. CTCL blood samples fractionated by FACS or MACS open up the opportunity to analyze those cells that are most likely involved in tumour genesis and progression.

When specific alterations between the protein profiles are detected by ProteinChip technology, single peaks can be isolated and identified by either 2-DE or ProteinChip technology [11] and by collision-induced dissociation (CID) using a ProteinChip interface coupled to a tandem mass spectrometer [12].

In the study presented here, fractions of CD4+ and CD4− lymphocytes from 25 CTCL patients and 25 normal controls were analysed on ProteinChip Arrays, because it is known that CD4 positive lymphocytes are activated in CTCL. The resulting protein profiles were submitted to a clustering algorithm, a rule extraction and rule base construction step which together excluded the possibility of finding a protein pattern by chance. For the prediction of CTCLs, the generated combined rule base resulted in a specificity of up to 84% and sensitivity of 92% for the CD4+ cell fraction and 96% specificity and sensitivity for the CD4− fraction.

2. Materials and methods

2.1. *Processing of blood samples*

All blood samples of CTCL patients (n = 34) were obtained from the Clinic of Dermatology of the Friedrich-Schiller-University Jena or the Clinic of Dermatology in Dresden, Germany with informed consent. CTCL diagnostics were performed in accordance with the guidelines of the German Cancer Society (AMWF) and were based upon clinical investigations: ultrasound investigations of lymph nodes, spleen and liver; routine laboratory investigations including: a complete differential blood count, Sezary cell count, liver enzymes, lactat dehydrogenase, renal parameters and inflammatory parameters (Westergreen blood sedimentation rate, C-reactive protein); and histology of lesional skin, lymphnodes (when enlarged) and bone marrow (when indicated). In addition to hematoxylin-eosin stains, immunostains and PCR for T-cell receptor were performed. CTCL were classified according to EORTC recommendations. Classification of all evaluable patients, who were all active, are given in Table 1. Blood samples from healthy sex-matched donors were used as a control (n = 30). These normal samples (5 ml) were stimulated with phytohaemagglutinin (PHA) to activate lymphocytes. Blood samples were further processed not longer than 12 h after sampling.

2.2. *Separation of lymphocytes by MACS*

Ten ml of heparinized blood was quartered in aliquots of 2.5 ml, diluted with 2.5 ml phosphate buffered saline (PBS), layered onto 5 ml chilled Biocoll (density 1.077 g/ml, Biochrome AG, Germany) and centrifuged at 800g for 30 min (without brake) at room temperature. After this density gradient separation, the resultant opaque interface containing lymphocytes was carefully transferred with a syringe into a centrifuge tube and washed two times with PBS.

The washed cells were resuspended in 80 μl of PBS and stained with CD4 microbeads (Miltenyi) for 15 at 4 °C. The PBS washed cells were centrifuged at 600g for 10 min, washed and resuspended in 500 μl PBS-FCS. Afterwards the cells were separated on a miniMACS column which was placed in a MACS magnetic field (Miltenyi Biotec GmbH, Germany) according to the manufacturer's instructions. The non-retained cells containing all other lymphocytes except CD4+ is further referred to as the CD4− fraction. The magnetically retained cells were eluted after washing and are designated as the CD4+ cell fraction. Before and after MACS, the cell number was analysed with a Neubauer counting chamber.